Drivers of US toxicological footprints trajectory 1998-2013

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METABOLIC ENGINEERING AND SYNTHETIC BIOLOGY - SHORT COMMUNICATION

A regulated synthetic operon facilitates stable overexpression of multigene terpenoid pathway in *Bacillus subtilis*

Ingy I. Abdallah[1] · Dan Xue[1] · Hegar Pramastya[1,2] · Ronald van Merkerk[1] · Rita Setroikromo[1] · Wim J. Quax[1]

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Abstract

The creation of microbial cell factories for sustainable production of natural products is important for medical and industrial applications. This requires stable expression of biosynthetic pathways in a host organism with favorable fermentation properties such as *Bacillus subtilis*. The aim of this study is to construct *B. subtilis* strains that produce valuable terpenoid compounds by overexpressing the innate methylerythritol phosphate (MEP) pathway. A synthetic operon allowing the concerted and regulated expression of multiple genes was developed. Up to 8 genes have been combined in this operon and a stably inherited plasmid-based vector was constructed resulting in a high production of C_{30} carotenoids. For this, two vectors were examined, one with rolling circle replication and another with theta replication. Theta-replication constructs were clearly superior in structural and segregational stability compared to rolling circle constructs. A strain overexpressing all eight genes of the MEP pathway on a theta-replicating plasmid clearly produced the highest level of carotenoids. The level of transcription for each gene in the operon was similar as RT-qPCR analysis indicated. Hence, that corresponding strain can be used as a stable cell factory for production of terpenoids. This is the first report of merging and stably expressing this large-size operon (eight genes) from a plasmid-based system in *B. subtilis* enabling high C_{30} carotenoid production.

Keywords *Bacillus subtilis* · MEP · Carotenoids · Cell factory · Stability

Introduction

Bacillus subtilis is a gram-positive bacterium that is considered as a GRAS (generally regarded as safe) organism by the Food and Drug Administration (FDA). The bacterium possesses properties that render it suitable for metabolic engineering to develop it as a cell factory, such as its fast growth rate and capability to grow on cheap raw material like molasses [22, 23]. *B. subtilis* also has a broad metabolic potential, no significant bias in codon usage and a wide substrate range. Hence, it is suitable for expression of diverse kinds of proteins allowing *B. subtilis* to be engineered to produce a multitude of metabolites including riboflavin [3, 17, 34].

Terpenoids are considered as one of the metabolite groups that are important in pharmaceutical, food, and cosmetic industries. Different terpenoids have been exploited as food colorants, fragrances or drugs such as the antimalarial artemisinin and anticancer paclitaxel [1, 8, 37]. High demand for medicinal terpenoids and the low yield of isolation from their natural sources warrant an alternative supply strategy [2, 6]. Microbial terpenoid cell factories have become one of the alternative choices in fulfilling the gap between demand and supply of these metabolites [2, 20]. Terpenoids are produced through two generic pathways called methyl erythritol phosphate (MEP) and mevalonate (MVA) pathway. *B. subtilis* has an innate MEP pathway (Fig. 1) with the capability to produce higher isoprene amounts compared to most eubacteria including *E. coli* [13]. Thus, it has the

Ingy I. Abdallah, Dan Xue and Hegar Pramastya have contributed equally to this work.

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✉ Wim J. Quax
w.j.quax@rug.nl

[1] Department of Chemical and Pharmaceutical Biology, Groningen Research Institute of Pharmacy, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands

[2] Pharmaceutical Biology Research Group, School of Pharmacy, Institut Teknologi Bandung, Bandung 40132, Indonesia

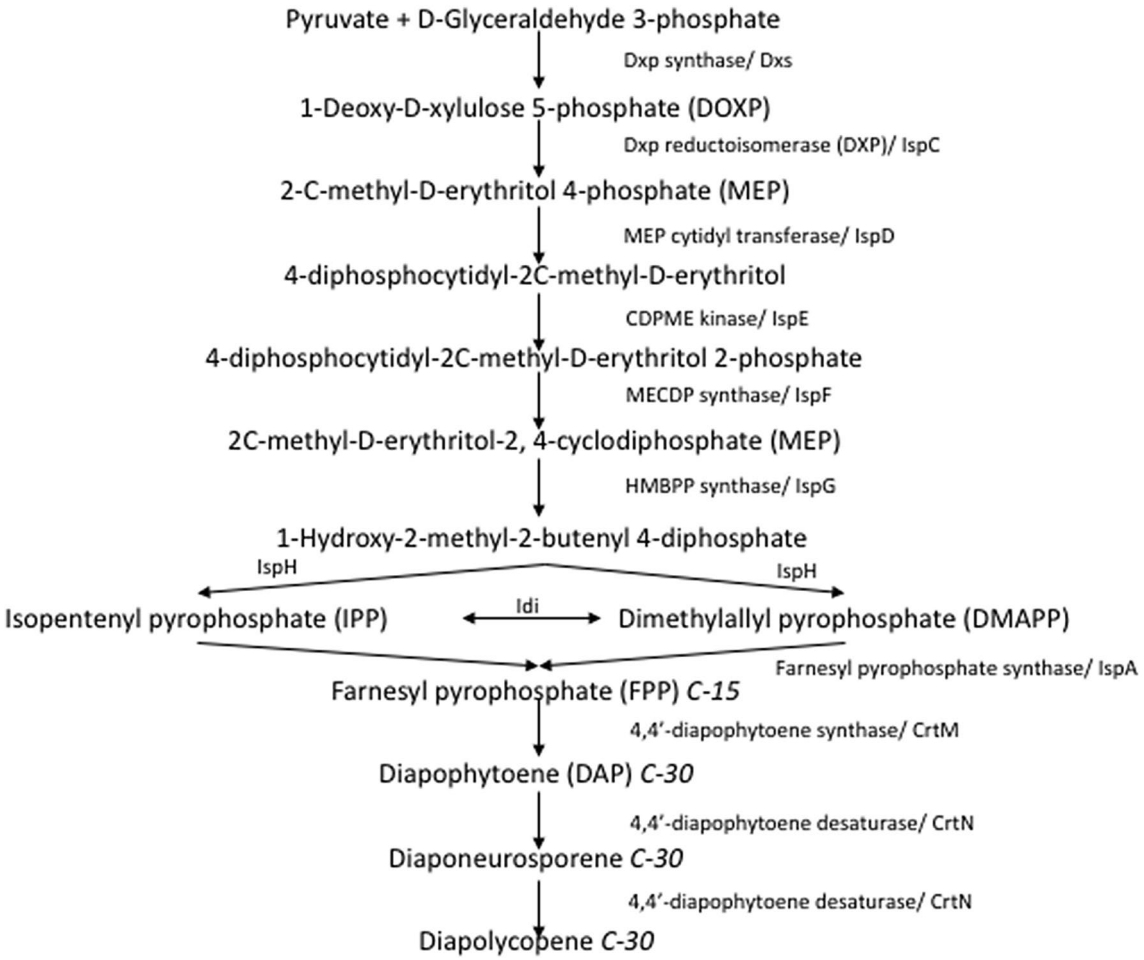

Fig. 1 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway

prospective to be engineered for high productivity of terpenoids. Further improvement of *B. subtilis* terpenoid production capability requires multigene overexpression of the MEP pathway enzymes. Tightly regulated overexpression of the MEP pathway genes is required to overcome toxicity due to intermediate product accumulation such as dimethylallyl diphosphate (DMAPP), isopentenyl diphosphate (IPP), and farnesyl diphosphate (FPP) at the beginning of the bacterium growth phase [26]. This regulation can be achieved using inducible promoters [5, 9]. Furthermore, there are also efforts to develop genetic manipulation tools of *B. subtilis*, particularly at the genomic level [7, 31, 33]. Nevertheless, for practical reasons and multicopy gene amplification ability, plasmids are still a choice for protein overexpression. In addition, endogenous genes of MEP pathway are spread on multiple locations under different regulatory elements in the *B. subtilis* genome [10]. Hence, constructing a single synthetic operon of MEP pathway genes under the control of an inducible promoter in a stable replicative plasmid would be beneficial.

Closest prior art on metabolic engineering of *B. subtilis* involving an operon of multiple genes contained only two genes in a rolling circle plasmid without having the stability data and manipulation on ribosome binding site to optimize the protein expression [40]. However, the stability of these rolling circle replication plasmids is usually poor prohibiting the scale up to fermentation status [4, 18, 25]. Hence, the need for a stable expression system in *B. subtilis* allowing the construction of plasmids with large inserts encompassing multiple genes is a pressing issue.

In this paper, we aim to bring together all of the endogenous MEP pathway genes in a regulated synthetic operon on a single plasmid facilitating high precursor supply for C_{30} terpenoid production. The validity of this approach is demonstrated by an unprecedented high production of C_{30} terpenoids by the concerted overexpression of the whole MEP pathway of *B. subtilis*. This system can serve as a basis for using *B. subtilis* as a cell factory for various commercially important terpenoids.

Materials and methods

Bacterial strains, growth conditions and vectors

Bacterial strains and expression vectors used in this research are listed in Table S1. Growth conditions are similar to our previous study [37].

Cloning strategy

Genes encoding *dxs*, *ispD*, *ispH*, *ispF*, *ispC*, *ispE*, *ispG* and *ispA* were amplified from *B. subtilis* genomic DNA, based on published annotation of its genome, using PCR with the suitable primers available in our previous study [37]. In our previous study, we were able to construct operons containing up to four genes of MEP pathway in pHCMC04G designated as SDFH (containing *dxs-ispD-ispF-ispH*), and CEGA (*ispC-ispE-ispG-ispA*) operons. To create the pHCMC04G construct containing seven genes of MEP pathway along with *ispA* gene, responsible for producing farnesyl pyrophosphate, the CEGA subset was amplified using primers (F-CEGA and R-CEGA as in Table S2 Supplementary Materials) that introduce overlapping flanks with the *Bgl*II-restricted p04SDFH construct. Subsequently, the CEGA was inserted to the restricted p04SDFH using circular polymerase extension cloning (CPEC) [21], resulting in p04SDFHCEGA (Fig. S1).

Expression of the genes from the different constructs in *B. subtilis* 168

With his-tag sequence at the last protein of the operon, we could check the expression of the respective protein before the downstream addition of another gene. *B. subtilis* 168 strains with pHB201 and pHCMC04G constructs were cultured then followed by preparation and detection of protein samples as previously published [37].

Real-time quantitative PCR (RT-qPCR) analysis

Bacillus subtilis 168 strain p04SDFHCEGA was incubated as described under 2.3. After induction with xylose, the culture was incubated further for 5 h and harvested for total RNA isolation. The total RNA was extracted from the pellet using Maxwell® 16 LEV simplyRNA Purification Kit with an additional enzymatic digestion step. The reverse transcription reaction was then performed immediately using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Promega) together with random primer (Promega) to synthesize cDNA. The thermal program was: incubate for 10 min at 20 °C, 60 min at 37 °C, 12 min at 20 °C, 5 min at 99 °C, and then keep the program at 4 °C. cDNA was used in qPCR immediately, or stored at − 20 °C until use.

Transcriptional level of target genes was analyzed by RT-qPCR with SYBR Green (SensiMix™ SYBR Low-ROX kit, Bioline) in QuantStudio™ 7 Flex Real-Time PCR System (Thermo Fisher Scientific). Each sample was measured in triplicate. The thermal cycling program was: 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 60 °C for 25 s, and followed by melting curve analysis using the defaulted program. Data analysis was carried out using QuantStudio™ Real-Time PCR Software v1.3 (Thermo Fisher Scientific). The p04SDFHCEGA plasmid was used to construct standard curves for quantitative analysis. The logarithmic of absolute copy number of each target part was interpolated from the standard curves. Primers were designed using NCBI Primer-BLAST online (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) [39]. The primers were designed to overlap between two genes at the beginning, middle and end of the operon and thus not to react with non-episomal genes. Hence, the primers used overlap as follows, between genes *dxs* and *ispD* (SD), between genes *ispH* and *ispC* (HC), and between genes *ispG* and *ispA* (GA), respectively.

Analysis of segregational and structural stability of the constructs in *B. subtilis* 168

Segregational stability was measured by evaluating the growth of *B. subtilis 168* cells harboring the p201SDFH, p201CEGA, p04SDFH, p04CEGA or p04SDFHCEGA constructs in TSB medium for 100 generations in the absence of antibiotics.

The cells of *B. subtilis 168* were first grown in 1-ml TSB broth containing 5 µg/ml chloramphenicol for 16 h at 37 °C. The overnight cultures were inoculated into 10-ml fresh TSB broth without chloramphenicol and incubated at 37 °C, 220 rpm for 24 h, attaining full growth. The cultures were diluted 1:1000 by fresh TSB broth without chloramphenicol and further incubated for 12 h (growth of 1:1000 dilution accounts for about 10 generations of cultivation, $2^{10} = 1024$). These cultures were diluted 10^6 fold and plated onto LB agar plates without chloramphenicol. After incubation at 37 °C overnight, 160 colonies were picked up and transferred onto LB agar plate supplemented with 5 µg/ml chloramphenicol. This treatment, starting from 1:1000 culture dilution followed by plating, was successively repeated 10 times to obtain 100 generations of cultivation. The presence of the plasmids was confirmed by the growth of the colonies on the plates, thus indicating that the plasmid hosted by the colonies is segregationally stable. The segregational stability of each construct was represented as % of colonies retaining the plasmid construct which is equal to [colonies on LB

plate with antibiotic/colonies on LB plate without antibiotic × 100%].

Structural stability of the constructs was determined as described above apart from the addition of chloramphenicol throughout the cultivation then colony PCR was used to detect large fragment deletions, in addition to random sequencing to detect mutations and small fragment deletions. Colony PCR was conducted using a pair of sequencing primers (Table S2) attached to the upstream and downstream of the operon of p201 and p04 constructs. The amplicons were then subjected to gel agarose electrophoresis to detect any large fragment deletions when a band smaller than the expected size was observed on the gel.

Production of carotenoids in *B. subtilis* strains overexpressing MEP pathway genes

The *B. subtilis* strains containing both pHB201 and pHCMC04G constructs of MEP pathway genes were transformed with the pHYCrtMN plasmid, bearing genes responsible for carotenoid production. The genes were expressed, and carotenoids were extracted and quantified as described in a previous study [37].

Nucleotide sequence accession number

The nucleotide sequence of the complete genome of *Bacillus subtilis* 168 is reported with the following accession numbers: AL009126 and NC000964. The MEP pathway genes used in this study were amplified from the genomic DNA of *B. subtilis* 168.

Results and discussion

Constructing synthetic operons harboring MEP pathway genes

The cloning strategy reported here allowed the insertion of multiple genes into a single synthetic operon controlled by the same promotor. Two vectors with different promoters and mode of replication were compared. pHB201 rolling circle replication vector with the P59 constitutive promotor and pHCMC04G theta-replication vector with xylose inducible promotor. The constructed operons contained genes of the MEP pathway starting from one gene up to eight genes (Table S3). It also permitted to insert before each gene the *B. subtilis* mntA ribosomal binding site, which is considered as a strong Shine-Dalgarno sequence ($\Delta G > 50.4$ kJ mol^{-1}). A spacing of six nucleotides to the starting codon was employed to ensure translational efficiency [32]. The presence of a C-terminal his-tag code at the end of the operon made it possible to purify the terminal protein encoded by each operon and evaluate its expression on Western blot using anti-his antibodies (Fig. 2a). This strategy allowed for consecutive insertion of genes where the expression of each inserted gene is confirmed before adding the next gene. All genes in each operon were checked by sequencing demonstrating that all transcripts are intact. Current cloning strategy involving CPEC method allowed us to put together seven genes of MEP pathway in addition to *ispA* into a theta-replicating plasmid.

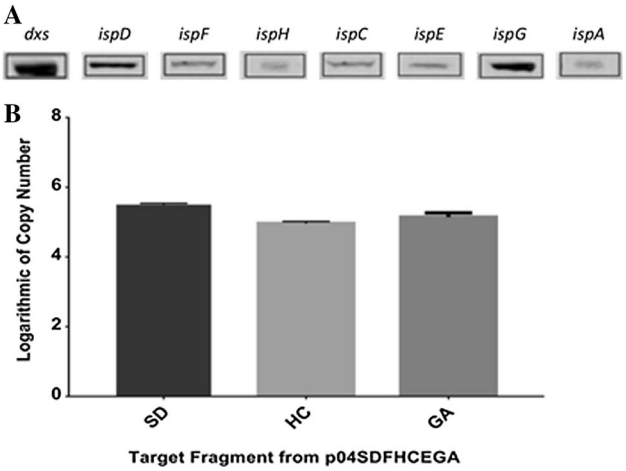

Fig. 2 **a** Western blot of pHCMC04G constructs of MEP pathway proteins expressed in *B. subtilis* 168. Proteins were isolated from *B. subtilis* 168 cell lysates and purified using His SpinTrap™ columns (GE Healthcare). After purification, protein samples were loaded on an SDS-gel and detected on Western blot using specific antibody against the his-tag. Volume of the samples was adjusted according to the concentration of each sample measured by nanodrop spectrophotometer for 40 µg of total protein. Dxs (70 kDa); IspD (26 kDa); IspF (17 kDa); IspH (35 kDa); IspC (43 kDa); IspE (32 kDa); IspG (41 kD); IspA (32 kDa). The differences in intensities of the bands maybe influenced by the differences in availability of the his-tag for SpinTrap™ and antibody binding. The proteins are all translated from the same transcript using the same RBS. **b** Expression level of genes in *B. subtilis* 168 containing p04SDFHCEGA construct. The expression level of each target fragment was represented as the logarithmic of absolute copy number per unit input total cDNA (10 ng), quantified by qPCR using serial dilutions of standards. SD represents beginning of the operon, fragment containing overlap of genes *dxs* and *ispD*; HC depicts middle of the operon, fragment containing overlap of genes *ispH* and *ispC*; GA illustrates end of the operon, fragment containing overlap of genes *ispG* and *ispA*. Mean values of three independent experiments with standard deviation are indicated by error bars

Study of p04SDFHCEGA operon expression using RT-qPCR analysis

The p04SDFHCEGA strain contains eight genes in the same operon regulated by a single promotor. RT-qPCR analysis was used to confirm equal expression level of all genes in the operon. Since the *B. subtilis* genome contains a copy of each of the MEP pathway genes, using primers specific for

each gene would not differentiate between the expression levels of the chromosomal and plasmid genes. Hence, primers overlapping at sequences between two genes at the beginning (SD), middle (HC) and end (GA) of the operon were designed to avoid amplifying the chromosomal genes. This is indeed confirmed by the absence of any signal in the wild-type strain. The expression level of each target fragment is represented as the logarithm of absolute copy number per unit input total cDNA. The level of expression of the genes at the beginning, middle and end of the p04SDFHCEGA operon was nearly similar (Fig. 2b) indicating that the single promotor was effective in controlling the expression of the entire operon in pHCMC04G vector. The results show that the transcripts are intact and all genes are expressed at the same level irrespective of their position in the operon. This can eliminate any doubt about the effect of the long length of the operon on the integrity of the mRNA transcripts and in turn, on protein expression.

Segregational and structural stability of the constructed plasmids in *B. subtilis* 168

After the successful compilation of the operons, a comparison between the pHB201 and pHCMC04G constructs was made investigating their segregational and structural stability in *B. subtilis* 168. The pHCMC04G strains show approximately 100% ability to retain the plasmid construct in medium without antibiotic until the 40th generation after which a slight loss of the plasmid occurred (Fig. 3). The plasmids showed over 85% stability inheritance until the 100th generation. In contrast, the strains with rolling circle plasmid pHB201 showed significant loss starting from the 20th generation ending with more than 70% plasmid loss

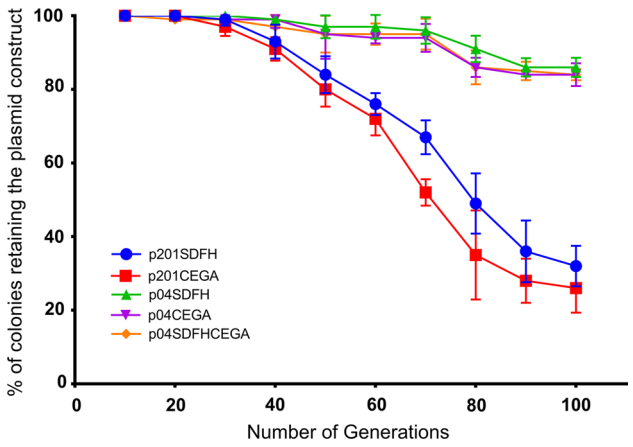

Fig. 3 Segregational stability of pHB201 and pHCMC04G constructs in *B. subtilis* 168. The stability of strains was represented as the % of colonies retaining the plasmid formed on the chloramphenicol-containing plates after successive subculturing (100 generations) from three different independent cultures

by the 100th generation. Sequencing of the pHCMC04G constructs indicated that 100%, 88% and 90% of the colonies of p04SDFH, p04CEGA and p04SDFHCEGA strains, respectively, possessed the correct sequence of the operon after 100 generations indicating their structural stability. In contrast, the sequences of the constructed operons in pHB201 usually showed deletions and mutations when the plasmid size became more than 10 kb based on colony pcr and sequencing performed. Sequencing results showed that already after the 10th generation only 57% and 62% of the colonies of p201SDFH and p201CEGA strains, respectively, had the correct sequence. Note that, the creation of a pHB201 eight gene construct turned out to be impossible further proving the segregational and structural instability of the pHB201 constructs.

These results are in line with the facts that rolling circle replication plasmids usually suffer from structural instability where recombination of short direct repeats present within this single-stranded DNA may lead to the deletions [12, 18, 24]. In addition, pHB201 is a plasmid that lacks active partitioning during replication which makes it prone to segregational instability causing loss of the entire plasmid population from a cell. It is noteworthy to mention that the instability of pHB201 is also observed independent of the use of a strong constitutive promotor, as pHB201 showed the same pattern of instability when a xylose promotor was used. It has been described that more stable plasmids might be derived on the basis of theta-replication mechanisms, originated from large plasmid of gram-positive bacteria such as pAMβ1 from *Streptococcus faecalis*, pTB52 from thermophilic *Bacillus,* and also pLS20 and pLS32 from *B. subtilis* natto [11, 19, 28–30]. Theta-replication-based plasmids are mostly low-copy number plasmids that replicate in the host through a theta-type intermediate where two replication forks proceed independently around the DNA ring; hence, they are structurally and segregationally stable up to a size of 50 kb compared to rolling circle replication plasmids in which nucleic acid replication is unidirectional leading to instability [15, 19, 24, 27]. Our results are fully in line with these findings as we show that even a metabolically very active synthetic operon can be stably maintained.

Enhanced carotenoid production in *B. subtilis* strains overexpressing MEP pathway genes

The OD_{600} of all MEP pathway engineered *B. subtilis* strains after 24-h growth ranged from OD_{600} 7–9. The total amount of carotenoids, 4,4′-diaponeurosporene and 4,4′-diapolycopene, produced in the different *B. subtilis* strains overexpressing MEP pathway genes with the help of pHB201 or pHCMC04G constructs was calculated as (mg/g dcw) to allow comparison between the strains. As a control, *B. subtilis* strain that only contains the pHYCrtMN plasmid

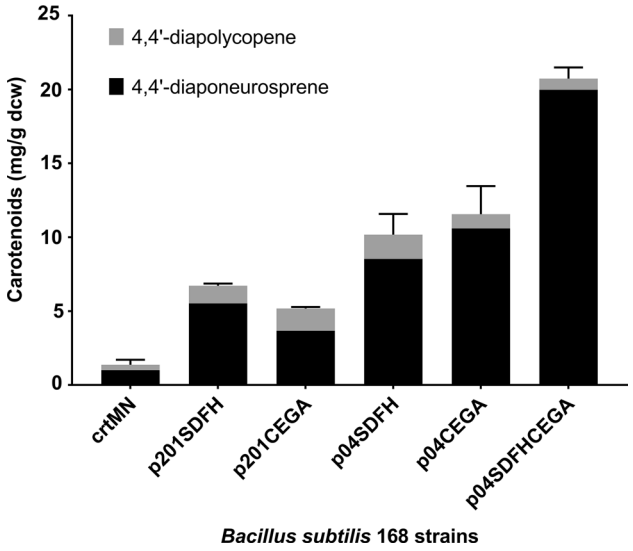

Fig. 4 Total amount of carotenoids produced by *B. subtilis* 168 strains containing pHB201 or pHCMC04G constructs overexpressing MEP pathway genes in addition to pHYCrtMN construct. The amount of carotenoids was represented as mg/g of dry cell weight

was used. The amount of total carotenoids produced in the *B. subtilis* strains containing pHB201 constructs overexpressing MEP pathway genes is less than that produced by the strains containing pHCMC04G constructs by approximately 50% (Fig. 4); this is in accordance with the decreased stability of the pHB201 constructs compared to the pHCMC04G constructs. The pHCMC04G strain overexpressing the eight genes, *dxs*, *ispD*, *ispH*, *ispF*, *ispC*, *ispE*, *ispG*, and *ispA* showed the highest amount of carotenoids produced, approximately 21 mg/g dcw, around 20-fold higher than the control strain with only pHYCrtMN plasmid. This amount of C_{30} carotenoids produced has never been reported before. The production level in *B. subtilis* can compete with production of C_{40} carotenoids such as lycopene reported in *E. coli* at 7.55 mg/g dcw [42] and at 24.41 mg/g dcw in *Saccharomyces cerevisiae* [35], β-carotene at 20.79 mg/g dcw in *S. cerevisiae* [36], zeaxanthin at 11.95 mg/g dcw in *E. coli* [14] or astaxanthin at 8.64 mg/g dcw in *E. coli* [16] and at 8.10 mg/g dcw in *S. cerevisiae* [41]. In addition, the reported production level in the eight gene *B. subtilis* strain is higher than that reported for other terpenoids in *B. subtilis* such as 0.5 mg/L of isoprene [38] and 17 mg/L of amorphadiene [40].

Conclusion

A *B. subtilis* strain overexpressing the whole MEP pathway (p04SDFHCEGA) in a stable manner was successfully created. This is the first report of the expression of the complete MEP pathway in a plasmid-based system in *B. subtilis* where it was proven that such a large operon can be stably expressed. This strain significantly improved the production of C_{30} carotenoids in *B. subtilis*. Together with its GRAS status, that *E. coli* does not possess, and fast growth rate, this could make *B. subtilis* a preferable cell factory for the production of different valuable terpenoids such as artemisinin and paclitaxel at the industrial level.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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